

# Acquisition of lethal doses of *Beauveria bassiana* conidia by western flower thrips, *Frankliniella occidentalis*, exposed to foliar spray residues of formulated and unformulated conidia <sup>☆</sup>

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Received 22 December 2004; accepted 5 July 2005

Available online 13 September 2005

## Abstract

Secondary acquisition of *Beauveria bassiana* conidia was recorded on the whole bodies and selected body parts of second-instar nymphs and adult female western flower thrips exposed to foliar spray residues of three differently formulated conidial preparations, for 24 h. Conidia were formulated in emulsifiable oil or with clay (wetttable powder), or were essentially unformulated conidia (technical grade powder suspended in water with a surfactant). Formulation had no significant effect on dose acquisition and no effect on virulence of acquired conidia. The mean nymphal LC<sub>50</sub>/LD<sub>50</sub> was 116 conidia/mm<sup>2</sup> and 52 conidia/insect, respectively; the values for adults were 19 conidia/mm<sup>2</sup> and 5 conidia/insect. Greatest numbers of conidia were recorded on the legs and abdomens of nymphs and on the legs, wings, and thoraces of adults. As would be expected, numbers of conidia acquired increased with residue concentration (application rate). However, an inverse relationship was noted between acquisition rate (conidia acquired/total conidia applied) and residue concentration. The mechanism underlying this response was not determined. However, there was no indication that any body parts (e.g., tarsi) became saturated with spores, which suggests that either the thrips were repelled by the conidial residues or that as the concentrations of conidia on the substrate increased, conidia somehow became more difficult to acquire. Slopes of the LC probit regressions were lower than those of the LD regressions (mean 1.14 vs 1.78), suggesting that the low slopes often obtained in fungal pathogen assays could be partly an artifact of unequal rates of dose acquisition at low vs high application rates.

Published by Elsevier Inc.

**Keywords:** Western flower thrips; *Frankliniella occidentalis*; *Beauveria bassiana*; Bioassay; Formulations; Foliar spray residues; Secondary pick-up

## 1. Introduction

The western flower thrips, *Frankliniella occidentalis* (Pergande), causes substantial economic losses to greenhouse crops throughout the world. The losses are generally

attributed to leaf, flower, and fruit deformation, and fruit/flower abortion caused by thrips feeding damage, plant death, or unsaleability due to infection with thrips-transmitted tospovirus. The ability of western flower thrips to develop resistance to chemical insecticides (Brødsgaard, 1994; Immaraju et al., 1992; Zhao et al., 1994) has led to the investigation of a variety of biological agents with thrips-control potential in greenhouse crops. These agents include the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin, (Brownbridge et al., 2000; Murphy et al., 1998; Vestergaard et al., 1999).

<sup>☆</sup> This paper reports the results of research only. Mention of a proprietary product does not constitute an endorsement or recommendation by the USDA.

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The fungal pathogen *B. bassiana* is available commercially for use against thrips in United States greenhouses as BotaniGard 22WP, BotaniGard ES, and Naturalis-L. Applications of these products have been reported to significantly reduce thrips populations in greenhouse cucumbers, chrysanthemums, gerbera daisies, roses, and carnations (Bradley et al., 1998; Jacobson et al., 2001; Ludwig and Oetting, 2002; Murphy et al., 1998; Shipp et al., 2002). It has recently also been confirmed that the active agent in the BotaniGard products, *B. bassiana* strain GHA, is highly virulent against *F. occidentalis* second-instar nymphs under laboratory conditions ( $LD_{50} < 50$  conidia/nymph) (Ugine, 2004).

Despite these promising experimental results, efficacy has been inconsistent under commercial production conditions, and *B. bassiana*-based biopesticides have not been widely adopted for thrips control. The reasons for the erratic performance of these mycoinsecticides are not known and research was consequently initiated to investigate potential efficacy-limiting factors.

Western flower thrips are thigmokinetic and thus typically inhabit parts of plants that are shielded from spray applications. Since direct targeting of thrips can be difficult, efficacy of contact control agents such as fungal pathogens may be dependent upon the capacity of the target pest to acquire a lethal dose of infectious propagules (conidia) from treated surfaces of the host plant. Secondary acquisition of *Metarhizium* and *Beauveria* conidia from treated foliage has been established as an important mode of inoculation for many locust and grasshopper species (Inglis et al., 1996; Jenkins and Thomas, 1996; Langewald et al., 1997; Lomer et al., 1993), and there are numerous other examples of secondary acquisition playing an important or potentially important role in fungal efficacy (Fernandez et al., 2001; Roditakis et al., 2000; Ugine, 2004).

Formulation technology is widely viewed as having great potential to improve the efficacy of microbial biocontrol agents (Burgess, 1998). The broad range of contemporary mycoinsecticide formulations, based on aqueous or oil carriers and incorporating surfactants, emulsifiers, and other adjuvants, have the potential to influence secondary dose acquisition.

The thrips-control efficacy of *B. bassiana* reported in the above cited studies was achieved using two distinctly different types of formulations, emulsifiable oils (EO) and wettable powders (WP). There is no conclusive evidence that either of these formulations has superior activity against thrips. Murphy et al. (1998) concluded that the EO formulation of BotaniGard (described as an emulsifiable suspension, ES, by the manufacturer) was more rapidly efficacious than the WP, whereas Bradley et al. (1998) reported faster action of the WP.

A number of laboratory and field studies indicate that oil formulation improves the efficacy of fungal pathogens (see review by Inglis et al., 2002). The enhanced

efficacy is generally attributed to the fact that oils are excellent stickers, promoting contact between the formulated active ingredient and the lipophilic insect cuticle and increasing rain-fastness on the waxy leaf cuticle of treated host plants. Yet, little is known about how oil formulation affects secondary acquisition of inoculum. In particular, it is not known if the perceived advantage of increased foliar rain-fastness might be disadvantageous by reducing secondary pick-up. Such questions have important implications for the formulation of fungal pesticides for the control of pests exhibiting broad ranges of behaviors.

In a previous study, it was demonstrated that western flower thrips readily acquired lethal doses of *B. bassiana* conidia from treated leaf surfaces (Ugine, 2004). Conidia in that study were minimally formulated (i.e., suspended in water with a low concentration of wetting agent). In the current study, we expand these investigations to examine and compare secondary acquisition of conidia from leaf surfaces treated with different formulations of this fungal pathogen. We were specifically interested in determining how fungal formulation might affect: (1) secondary acquisition of conidia on the whole body and specific body parts of western flower thrips, (2) inoculum acquisition rates, and (3) fungal virulence. Experiments were conducted using two commercially available formulations (one EO and one WP) and an unformulated technical grade powder (TGP) of *B. bassiana* strain GHA.

## 2. Materials and methods

### 2.1. General methods

#### 2.1.1. Rearing and selection of thrips for bioassays

Western flower thrips adults collected from a Cornell University research greenhouse were used to establish a colony. Thrips were continuously reared on excised leaves of red kidney beans (*Phaseolus vulgaris* L.) in small plastic containers (16 cm × 16 cm × 6 cm) with snap-on lids. A hole (10.5 × 10.5 cm) was cut in the lid and covered with thrips-proof organdy (mesh openings of 95 µm) (Sefar America, Kansas City, MO), to allow for ventilation. Containers were maintained at 25 ± 1 °C under a 14:10 h light:dark regime.

Adult female western flower thrips (25–30) were added to a rearing container and allowed to oviposit for 24 h. Five and eleven days after initial infestation of a rearing container, the thrips population comprised primarily second-instar nymphs and newly emerged adults (<24 h old), respectively. From this population, thrips were collected via aspiration into 1 ml centrifuge tubes. The vials, each containing groups of 12 nymphs or 6 adult females, were then randomly assigned to the treatments associated with an assay. For additional details of the rearing/selection processes see Ugine (2004).

### 2.1.2. Fungal preparations

Conidia of *B. bassiana* strain GHA were produced and formulated by Emerald BioAgriculture (Butte, MT) using proprietary methods and ingredients. The technical grade (unformulated) powder (TGP), the clay-based WP (wetttable powder) formulation (BotaniGard 22WP), and the paraffinic oil-based EO (emulsifiable oil) formulation (BotaniGard ES) were stored under refrigeration (4°C) after receipt from the manufacturer.

Stock suspensions were prepared by mixing each of the fungal preparations in deionized water. Silwett L-77, an organosilicone surfactant (Loveland Industries, Greeley, CO), was added at a concentration of 0.01% to suspend the TGP; the WP and EO formulations contained surfactants/emulsifiers and Silwet was not added. Aliquots of 30 ml water or water + Silwet solution were prepared in 50-ml plastic centrifuge tubes. One gram of glass beads (2 mm diameter) was added to the tubes receiving the dry powder preparations and the tubes were agitated on a wrist action shaker (Model BT, Burrell Scientific, Pittsburgh, PA) set at maximum speed (6.7 oscillations/s) for 15 min. Suspensions of the EO formulation were prepared with minimal agitation (mixing by hand), as the mechanized agitation protocol used for the powder formulations resulted in destabilization of the emulsion (the oil droplets coalesced and adhered to the walls of the centrifuge tube).

### 2.1.3. Bioassay protocol

Leaf disks (2 cm diam.) were cut from the first true leaves of red kidney bean plants and the abaxial surfaces were spray treated with the appropriate conidial preparation using a Burgerjon spray tower (Burgerjon, 1956). The tower was fitted with an air-atomizing nozzle (Fluid Cap 2850 + Air Cap 70) mounted in a 1/4J nozzle body (Spraying Systems, Wheaton, IL) connected to a regulator valve providing a constant airflow of 10 liter/min. Spray targets were positioned on a rotating turntable (33 rpm) during application, and spray deposition at the level of the target surface was approximately 0.01  $\mu\text{l}/\text{mm}^2$  (resulting from spraying of a 5 ml aliquot). Two leaf disks, which were allowed to dry before being added to 30 ml portion cups, were placed with their abaxial surfaces together, between two filter paper disks. Each piece of filter paper was moistened with 35  $\mu\text{l}$  deionized water. For a more detailed description of the leaf-disk application bioassay and procedures, see Ugine (2004). Randomly selected groups of 12 early second-instar thrips, or 6 newly emerged adult-female thrips were added to an assay cup, which was then sealed with parafilm and capped with the plastic snap-on lids provided by the manufacturer.

### 2.1.4. Quantification of spray application rates and acquired doses

Numbers of conidia deposited on the leaf disks were estimated from sample deposits collected on polystyrene

Petri dish lids (90 mm diam.) placed on the target platform of the spray tower during each spray application. In order to quantify the conidia, a drop of lactic acid (85%) containing acid fuchsin (1 mg/ml), was placed onto the Petri dish lid, covered with a coverslip, and viewed at 400 $\times$  magnification. Numbers of conidia per  $\text{mm}^2$  were determined using the procedure described by Wraight et al. (1998), except that 5 replicate counts were made per dish lid.

The number of conidia acquired on both the dorsal and ventral surfaces of adult and second instar thrips were enumerated following the protocol described by Ugine (2004). Thrips were mounted in lactic acid (85%) containing acid fuchsin (1 mg/ml) and lightly compressed between two glass cover slips taking care not to rupture the body wall. They were then examined under a phase contrast microscope at 400 $\times$  magnification. Conidia stained red and were recorded as having been observed on one of five or six body parts (head, mouthparts, thorax, legs, wings, or abdomen) depending on thrips life stage. Both the dorsal and ventral surfaces of each thrips were examined. Conidia-like objects on spray-carrier treated thrips (carrier controls) were also quantified and the mean numbers of conidia on fungus-treated insects were corrected for these 'background' numbers by subtracting the mean number of conidia-like objects from the count for each insect. Numbers of these objects were low in all cases (<4 per thrips).

Additionally, a Petri dish with yeast extract agar (1%) was sprayed along with the leaf disks in each test. The inoculated plates were sealed with parafilm and incubated for 24 h at 25  $\pm$  1 °C. The spray deposits were then stained with acid fuchsin, and the first 100 conidia encountered under phase contrast microscopy (400 $\times$ ) were scored for germination. This procedure was conducted at five randomly chosen locations per Petri plate and numbers of conidia in all assays were corrected for viability.

## 2.2. Virulence bioassays

The comparative virulence of the three formulations against second-instar thrips was determined in a series of four-dose bioassays. The TGP was applied to bean leaf disks as aqueous suspensions containing 0.01, 0.025, 0.05, and 0.10 mg powder/ml. These concentrations were selected to produce a range of depositions from ca. 10 to 150 viable conidia/ $\text{mm}^2$ . The WP was applied at concentrations of 2.21, 1.11, 0.221, and 0.0221 mg powder/ml and the EO was applied at concentrations of 5.17, 2.59, 0.517, and 0.0517  $\mu\text{l}/\text{ml}$ . These concentrations were predicted to produce depositions ranging from ca. 10 to 1000 conidia/ $\text{mm}^2$ .

A total of six replicate cups were assembled for each application rate (72 nymphs/rate). The bioassay of each formulation was conducted a minimum of four times over

a one month period using different cohorts of thrips from the laboratory colony. A total of 16 assays were conducted; however, in three of these, mortality was too low for estimation of median lethal concentrations or doses. Each assay included six replicate cups of thrips exposed to the appropriate spray carrier containing no fungal conidia (carrier controls). For the TGP assays, the control leaf disks were sprayed with Silwet at 0.01% (v/v); for the WP and EO assays, controls were treated with carrier blanks provided by the manufacturer at application rates equivalent to the highest rate applied in the assay.

The WP formulation was also assayed against adult-female thrips following the protocol described for nymphs except that only six adult thrips were introduced into each cup (36 adults/rate). A series of four adult assays was conducted ca. 5 months after completion of the nymphal assays.

Assay cups were incubated at  $25 \pm 1^\circ\text{C}$  in a growth chamber under a natural light regime (incubator with glass-door in a laboratory with unshaded windows). One cup from each application rate group was removed 24 h post-application and a sub-sample of five thrips was examined microscopically to determine the acquired dose. Dose was ultimately reported as the mean number of viable conidia per five nymphal or adult-female thrips. The remaining five cups were used to assess mortality 5 days post-treatment. The time of sampling (24 h post-application) was based on a previous study Ugine (2004), which revealed that conidia acquired by the second-instar thrips >24 h after treatment produced few additional infections, as it was presumed that these conidia were lost in the molt to the pupal stage. Adult females were also incubated for 24 h, despite the fact that conidial acquisition after 24 h could potentially contribute to mortality, given that adults do not molt. It was determined in preliminary assays that 24 h vs continuous exposure did not result in significantly different mortality rates within the timeframe of the bioassay (5 days).

### 2.3. Dose acquisition by body parts

Total numbers of differently formulated conidia acquired by second-instar thrips from treated leaf disks

and the relative virulence of these conidia was investigated through the above described bioassays. Secondary acquisition of conidia from the treated leaf disks was then further assessed on various parts of the thrips body. To assess formulation effects, acquisition was investigated on replicate groups of thrips that had been treated with similar rates of each formulation. This was achieved by segregating the bioassay units (cups) sampled 24-h post-treatment in the above described assays into three application rate categories (representing a low, medium and high rate), each with lowest possible variability (excluding some intermediate and extreme rates). Means (and ranges) of the rates (with 13, 14, and 8 replicates, respectively) were 13 (3–22), 70 (45–114), and 885 (592–1093) viable conidia/mm<sup>2</sup> (Table 1). Four to six replicate cups were selected per application rate per formulation with the exception that the TGP was not tested at the high rate. To provide maximum power for statistical testing of formulation effects, statistical independence of the replicates was maintained only within rate categories (i.e., each replicate within a rate category was selected from a different bioassay). All comparisons among formulations were then restricted within rates.

The acquisition of WP-formulated conidia by adult-female thrips was also investigated. Means (and ranges) of the low, medium–low, medium–high, and high rates (each comprising four replicates) were 11 (7–14), 69 (32–112), 285 (169–447), and 1880 (1147–2280) viable conidia/mm<sup>2</sup>. The assay was conducted four times, and in this case, each assay represented a complete block, enabling comparisons across application rates.

For both nymphal and adult thrips, acquisition was further evaluated in terms of conidia acquired per body part across formulations as well as per unit surface area of each body part, also across formulations. To examine these relationships among the second-instar assays, a protocol similar to that described previously was followed to produce three rate categories with minimal variability. In this case, however, maintaining statistical independence among replicates across application rates restricted selection to just one group from each of the 16 bioassays. Selections were made without regard to formulation (see results) and numbers used in the formula-

Table 1

Mean  $\pm$  SE number of *B. bassiana* conidia applied to leaf disks at three application rates of a technical powder, wettable powder, and an emulsifiable suspension

Formulation	Conidia/mm <sup>2</sup> at three application rates		
	Low	Medium	High
Technical powder	11.8 $\pm$ 2.9 ( <i>n</i> = 4)	73.0 $\pm$ 15.0 ( <i>n</i> = 4)	—
Wettable powder	12.6 $\pm$ 3.0 ( <i>n</i> = 5)	72.7 $\pm$ 7.9 ( <i>n</i> = 6)	900.0 $\pm$ 110.9 ( <i>n</i> = 4)
Emulsifiable suspension	13.4 $\pm$ 4.5 ( <i>n</i> = 4)	62.7 $\pm$ 7.5 ( <i>n</i> = 4)	870.0 $\pm$ 80.8 ( <i>n</i> = 4)
Grand mean	12.8 $\pm$ 1.8	70.1 $\pm$ 5.5	885.0 $\pm$ 63.8
ANOVA	$F_{[2,10]} = 0.05, P = 0.95$	$F_{[2,11]} = 0.31, P = 0.74$	$F_{[1,6]} = 0.05, P = 0.83$

Mean  $\pm$  SE application rate applied vs groups of 12 s instar thrips.

tion study were excluded. The resulting low, medium, and high rate categories (with 5, 6, and 5 replicates, respectively) had means (and ranges) of 5 (3–7), 63 (45–114), and 974 (787–1093) viable conidia/mm<sup>2</sup>.

Surface areas were estimated by translating the thrips body into a composite of regular geometric forms (cylinders or cones). Measurements were taken of the lengths and widths of the body parts of thrips using a dissecting microscope with an ocular micrometer and surface areas were calculated and expressed in units of 0.1 mm<sup>2</sup>.

#### 2.4. Dose acquisition rates

Finally, data from the above described studies were used to investigate the proportion conidial acquisition with acquisition rate defined as the number of conidia observed on the whole thrips body (or on a body part) divided by the total number of conidia to which the thrips were exposed (total conidia on the treated abaxial surfaces of the two leaf disks in the assay cups). As with conidial acquisition, acquisition rate was examined as a function of application rate and thrips body part.

#### 2.5. Statistical analyses

Median lethal concentrations and doses were estimated by probit analysis using the personal computer program POLO (LeOra Software, 1987). Treatment mortalities were corrected for control mortality by the POLO program. All other statistical tests were conducted using the JMP software (SAS Institute, 2002). Main effects and interactions were tested by standard ANOVA ( $\alpha = 0.05$ ). Conidial counts from the body parts of each individual thrips were analyzed as repeated measures using the MANOVA option in the JMP program; significance of interaction terms was tested using several alternative multivariate tests; reported *F* test results are those generated by the Pillai's Trace option. Fourteen MANOVAs were conducted which included body part and application rate as the main effects; among these, the interaction term was highly significant in only one case ( $P < 0.001$ ) and marginally significant in two other cases ( $P = 0.05$ ). The main effects of body part and application rate were therefore tested and interpreted without qualification.

Detection of a significant within-subject effect (main effect of body part) was followed by post hoc testing for means separation as recommended by Stevens (2002). The multivariate analysis platform provides estimates of the degree of deviation from the sphericity assumption, reporting both the Greenhouse–Geisser (G–G) and Huynh–Feldt (H–F) estimations of the  $\epsilon$  parameter. Tukey's HSD was considered valid for means testing in those cases where  $\epsilon$  (mean of the G–G and H–F values) exceeded 0.7 (see Stevens, 2002). The Tukey option was accessed via the JMP standard least squares: random

effects platform (standard univariate ANOVA, balanced split plot design). In all other cases, means were compared using multiple-dependent *t* tests (repeated measures MANOVA) with Bonferroni adjustments to maintain the experimentwise  $\alpha$  level at 0.05.

Acquisition of conidia appeared more or less random among the replicate batches of thrips within application rates and the square root transformation recommended by Sokal and Rohlf (1995) for data including low or zero values was therefore applied to conidial numbers prior to ANOVA (all variates coded by adding 0.5). The standard arcsine transformation was applied to data representing the percentages of conidia on the various body parts.

Ratios and rates are typically non-normally distributed. Plots of conidial acquisition rates vs spray application rates indicated a hyperbolic relationship and acquisition rates were transformed to normality for MANOVA by applying the reciprocal transformation (Sokal and Rohlf, 1995). To avoid zero values, the data were coded by adding 1.

### 3. Results

#### 3.1. Virulence bioassays

The three fungal formulations were equally efficacious against second-instar thrips. LC<sub>50</sub>s and LD<sub>50</sub>s were not significantly different among formulations (Table 2), indicating, also, no difference in acquisition of conidia from the treated substrate. Slopes of the LC regression lines (across all assays) were significantly lower than the slopes of the LD regressions (mean 1.14 vs 1.78;  $F_{[1,6]} = 6.2$ ;  $P = 0.048$ ).

Results of assays with the WP formulation indicated that the adult thrips were markedly more susceptible to fungal infection than the second-instar nymphs (LD<sub>50</sub> 5 vs 50 conidia/mm<sup>2</sup>, respectively). The slopes of the adult vs second-instar LD regressions did not differ (Table 2). A similar 6-fold difference was observed in the LC<sub>50</sub> values; however, the slopes of the adult LC regressions were significantly lower than those of the second instars (0.7 vs 1.3).

#### 3.2. Dose acquisition

Formulation also had no significant effect on the mean numbers of conidia observed on the five body parts of second-instar thrips (low application rate:  $F_{[2,10]} = 0.54$ ,  $P = 0.60$ ; medium rate:  $F_{[2,11]} = 1.5$ ,  $P = 0.26$ ; high rate:  $F_{[1,6]} = 0.26$ ,  $P = 0.63$ ; Fig. 1). There was a significant effect of body part within each of the application rates (low rate:  $F_{[4,7]} = 13.3$ ,  $P = 0.002$ ; medium rate:  $F_{[4,8]} = 55.8$ ,  $P < 0.0001$ ; high rate:  $F_{[4,3]} = 176.4$ ,  $P = 0.0007$ ). The formulation  $\times$  body part interaction

Table 2

LD<sub>50</sub> and LC<sub>50</sub> estimates from 4-rate bioassays against second-instar and adult-female thrips exposed to *Beauveria bassiana* conidia as a technical powder (TP), wettable powder (WP), and an emulsifiable oil (EO), in a leaf-sandwich bioassay<sup>a</sup>

	No. assays	LC <sub>50</sub> ± SE	Slope	χ <sup>2</sup> range <sup>b</sup>	Concentration range <sup>c</sup>	LD <sub>50</sub> ± SE	Slope	χ <sup>2</sup> range	Dose range <sup>d</sup>	Mortality range <sup>e</sup>
<i>Second instar</i>										
TGP	4	73.5 ± 16.1	1.28 ± 0.15	0.4–6.2	6–235	42.0 ± 10.5	1.34 ± 0.24	3.8–20.7	6–56	15–84% (9%)
WP	5	161.8 ± 35.1	1.27 ± 0.15	0.1–7.7	2–1093	63.4 ± 14.4	2.07 ± 0.42	0.3–11.1	3–266	11–93% (12%)
EO	4	111.9 ± 43.7	1.29 ± 0.16	0.01–8.4	3–1800	49.6 ± 9.9	2.17 ± 0.30	0.03–11.0	4–224	13–98% (14%)
ANOVA statistics <sup>f</sup>		F <sub>[2,10]</sub> = 2.03, P = 0.18	F <sub>[2,10]</sub> = 0.01, P = 0.99			F <sub>[2,10]</sub> = 0.91, P = 0.43	F <sub>[2,10]</sub> = 1.60, P = 0.25			
<i>Adult female</i>										
WP	4	19.0 ± 7.9	0.70 ± 0.10	1.6–20.9	7–2280	5.0 ± 2.6	1.48 ± 0.68	1.6–15.8	1–69	3–98% (10%)

<sup>a</sup> Median lethal concentrations and doses estimated from replicated four-rate bioassays (12 second-instar thrips/bioassay unit and 6 adult-female thrips/bioassay unit; 5 units/application rate). Mortality was recorded after incubation for 5 days at 25 °C.

<sup>b</sup> Heterogeneity X<sup>2</sup> value (Finney, 1971) with two degrees of freedom given by probit analysis.

<sup>c</sup> Range of concentrations (viable conidia/mm<sup>2</sup> of leaf-disk abaxial surface).

<sup>d</sup> Range of doses (viable conidia/insect). Doses were determined from direct counts of conidia on five insects/application rate/assay following 24 h exposure to treated leaf disks.

<sup>e</sup> Range of uncorrected percent mortalities experienced by second instar and adult thrips. Percentages in parentheses are the mean control mortalities for each group of assays.

<sup>f</sup> ANOVA statistics testing for differences in the mean LD<sub>50</sub>s, LC<sub>50</sub>s, and slopes among the three fungal formulations.

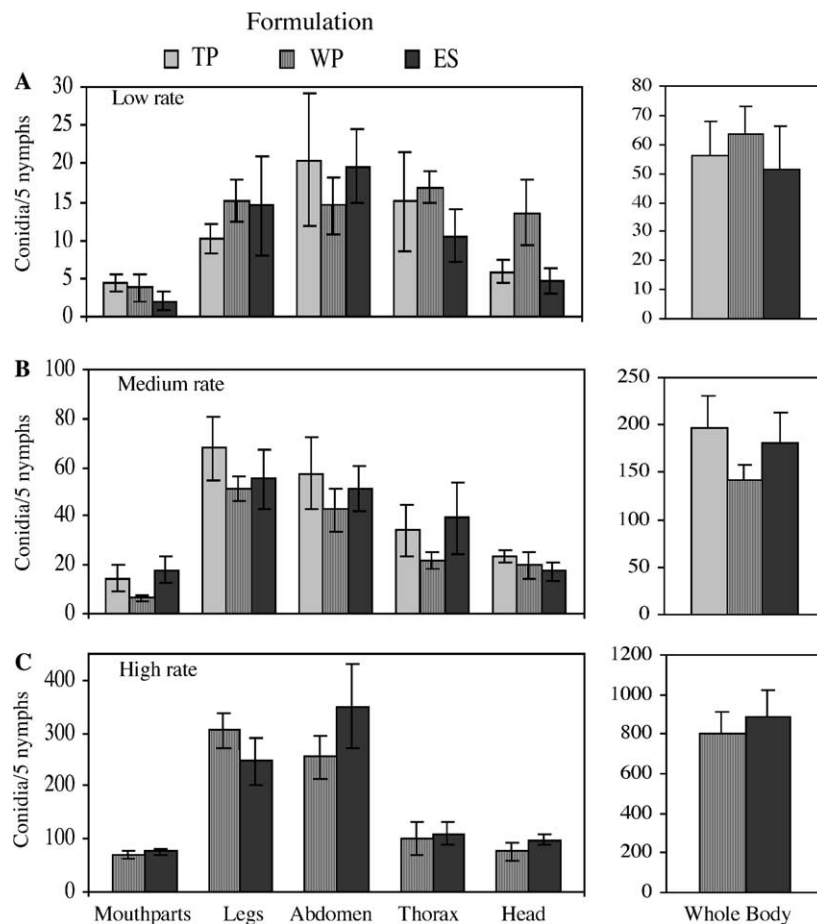


Fig. 1. Conidia on the whole body or indicated body parts per five second-instar western flower thrips exposed 24 h to bean leaf disks treated with a technical grade powder (TGP), wettable powder (WP), and emulsifiable oil (EO) formulations of *B. bassiana* at three application rates (13, 70, and 885 conidia/mm<sup>2</sup>); bars represent standard errors.

was not significant within any of the application rate groups (low rate:  $F_{[8,16]}=1.1$ ,  $P=0.41$ ; medium rate:  $F_{[8,18]}=0.50$ ,  $P=0.84$ ; high rate:  $F_{[4,3]}=0.58$ ,  $P=0.70$ ).

As formulation had no significant effect on dose acquisition, this factor was dropped from all subsequent analyses (i.e., examining distribution of conidia on the host and rates of conidial acquisition).

### 3.3. Distribution of conidia

The distribution of conidia on the different body parts of second-instar and female thrips varied significantly as a function of body part (main effect of body part) (nymphs:  $F_{[4,10]}=55.4$ ,  $P<0.0001$ ; adult females:  $F_{[5,8]}=22.7$ ,  $P=0.0002$ ; Figs. 2A and C). The greatest numbers of conidia were found on the legs and abdomen of second-instar thrips and the legs, wings, and thoraxes of adult thrips. The dose acquired across body parts of second-instar and adult-female thrips increased significantly as application rate increased (nymphs:  $F_{[2,13]}=85.2$ ,  $P<0.0001$ ; adult females:  $F_{[3,12]}=3.6$ ,  $P=0.045$ ). There were no significant application rate  $\times$  body part interactions for adult-female thrips (nymphs:  $F_{[8,22]}=2.2$ ,  $P=0.07$ ; adult females:  $F_{[15,30]}=1.7$ ,  $P=0.11$ ).

The distribution of conidia acquired per pair of second-instar and adult female legs (prothoracic, mesothoracic and metathoracic pairs) differed significantly as a function of application rate (nymphs:  $F_{[2,13]}=71.8$ ,  $P<0.0001$ ; adult females:  $F_{[3,12]}=5.8$ ,  $P=0.01$ ) and leg position for second instars but not for females (nymphs:  $F_{[2,12]}=4.0$ ;  $P=0.047$ ; adult females:  $F_{[2,11]}=0.38$ ,  $P=0.69$ ). The fewest conidia were acquired within the lowest application rates and the metathoracic legs acquired the fewest conidia on average compared to the remaining pairs. Additionally, there was a marginally significant application rate by leg interaction for nymphs (nymphs:  $F_{[4,26]}=0.92$ ,  $P=0.047$ ), but not for adult females (adult females:  $F_{[6,24]}=1.1$ ,  $P=0.40$ ). The trend of increased dose acquisition with increasing application rate remained quite pronounced (Fig. 3).

The designated body parts used to describe the distribution of conidia on thrips bodies differ greatly in total surface area; hence, it might be expected that those body parts with the smallest surface areas would acquire the fewest conidia. To directly compare conidial acquisition among body parts, conidial counts were therefore also determined on a per unit surface area basis. The adjusted numbers of conidia on the different body parts increased significantly as a function of application rate for second instars and while the numbers of conidia on adult females followed the same trend as that of second instars, the main effect of application rate was not significant (nymphs:  $F_{[2,13]}=88.1$ ,  $P<0.0001$ ; adult females:  $F_{[3,12]}=2.2$ ,  $P=0.09$ ) (Figs. 2B and D). There was once again a highly significant

main effect of body part for both life stages (nymphs:  $F_{[4,10]}=99.1$ ,  $P<0.0001$ ; adult females:  $F_{[5,8]}=33.2$ ,  $P<0.0001$ ); however a significant application rate by body part interaction was detected for second instar nymphs ( $F_{[8,22]}=9.5$ ,  $P=0.006$ ). The interaction was not significant for females ( $F_{[15,30]}=1.4$ ,  $P=0.24$ ). The numbers of conidia acquired by the body parts of second instars and females typically increased as application rate increased and the distribution of conidia among the different body parts was significantly altered from the original distribution once surface area was taken into account. Thus, those body parts with the smallest surface area, such as the head, legs, and mouthparts had more conidia per unit surface area ( $0.10\text{ mm}^2$ ) compared to large surface area body parts such as the thorax and abdomen (Figs. 2B and D). The distribution of conidia on the different body parts was further investigated by converting the numbers of conidia on each of the body parts into percentages relative to the total number of conidia acquired by the whole body (Figs. 4A and C). The main effect of application rate was not significant for either life stage (nymphs:  $F_{[2,13]}=1.3$ ,  $P=0.30$ ; adult females:  $F_{[3,12]}=1.6$ ,  $P=0.24$ ) and the relative percentages of conidia acquired across body parts did not differ significantly by application rate (no body part  $\times$  application rate interaction) (nymphs:  $F_{[8,22]}=2.2$ ,  $P=0.07$ ; adult females:  $F_{[15,30]}=1.4$ ,  $P=0.21$ ). The main effect of body part was highly significant for each life stage (nymphs:  $F_{[4,10]}=49.6$ ,  $P<0.0001$ ; adult females:  $F_{[5,8]}=33.7$ ,  $P<0.0001$ ). The highest percentages of conidia were attached to the legs and abdomens (29% and 32%) of second-instar thrips and the legs and thoraxes (46% and 26%) of adult females, while the lowest percentages of conidia were found on the mouthparts (7% and 2% on the mouthparts of nymphs and adults, respectively).

Percent acquisition on body parts was also examined with respect to total conidia per  $0.10\text{ mm}^2$  of the total body and per body part. Again, the main effect of application rate was not significant for either life stage (nymphs:  $F_{[2,13]}=2.0$ ,  $P=0.18$ ; adult females:  $F_{[3,12]}=2.1$ ,  $P=0.15$ ) (Figs. 4B and D). The interaction between application rate and body part was marginally significant for second instars (nymphs:  $F_{[8,22]}=2.4$ ,  $P=0.053$ ) and insignificant for adult females (adult females:  $F_{[15,30]}=1.4$ ,  $P=0.24$ ). The percentage of conidia acquired by the different body parts of second instars and adults differed significantly as a function of body part (nymphs:  $F_{[4,10]}=135.0$ ,  $P<0.0001$ ; adult females:  $F_{[5,8]}=172.7$ ,  $P<0.0001$ ). The mouthparts and heads of second instars (27% and 46%, respectively) and the legs and wings of adult females (33% and 26%) acquired the highest percentages of conidia per  $0.10\text{ mm}^2$ , whereas the abdomens of each life stage acquired the lowest percentages (4% and 2%, respectively) (Fig. 4).

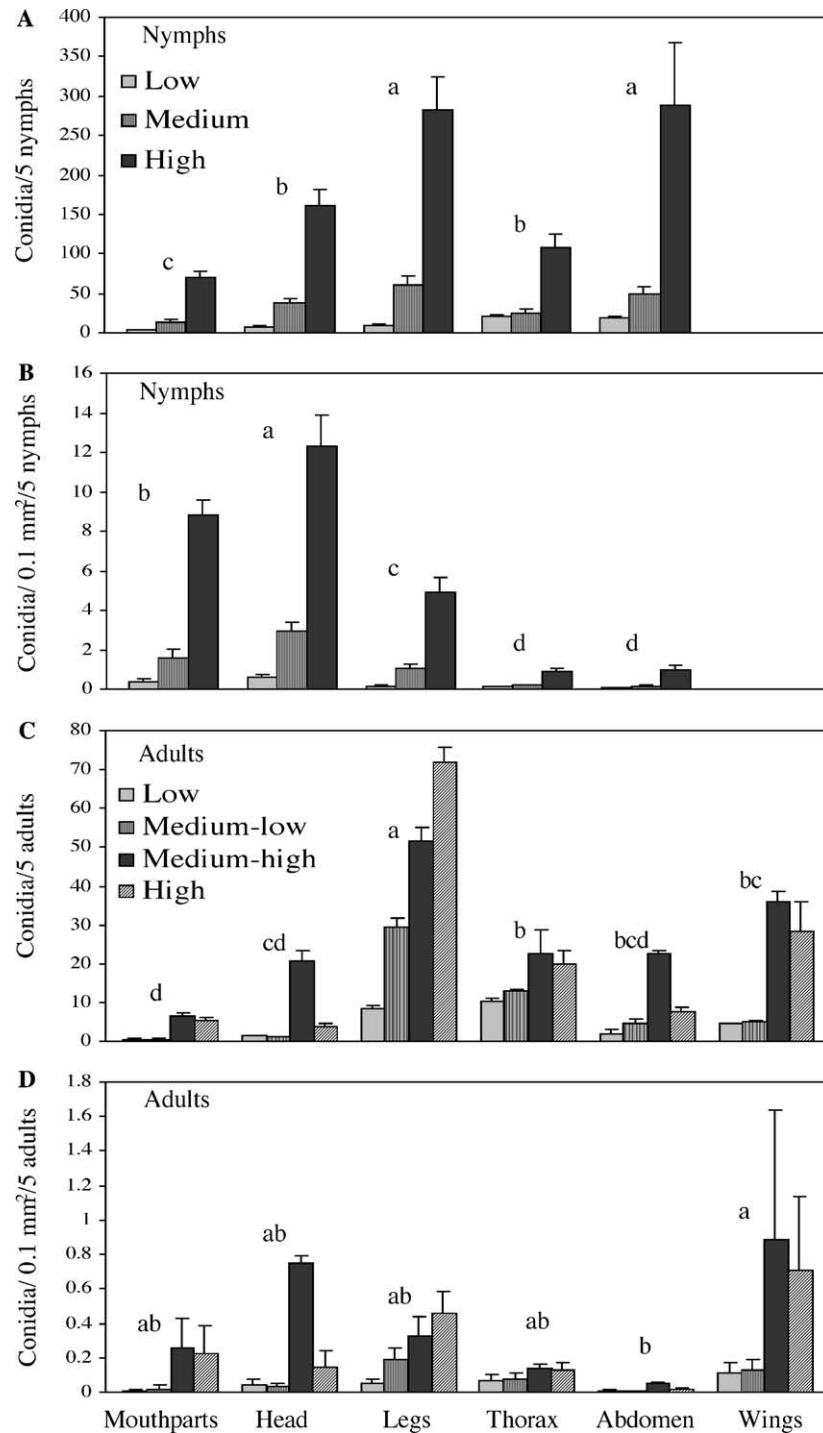


Fig. 2. Conidia per whole body part (A, C) and per unit surface area of each body part (B, D) per five second instar or five adult female western flower thrips exposed 24 h to bean leaf disks treated with *B. bassiana* at three or four application rates (5, 63, and 974 conidia/mm<sup>2</sup>; 11, 69, 285, and 1880 conidia/mm<sup>2</sup>, respectively); bars represent standard errors. Means (across application rates) with the same letter are not significantly different (A and C: Tukey's HSD test;  $\alpha = 0.05$ ; B and D: Bonferroni-adjusted *t* tests;  $\alpha = 0.05$ ).

### 3.4. Conidial acquisition rates

The rate of acquisition of conidia was low. Second-instar and adult-female thrips acquired, on average, only  $0.9 \pm 0.3\%$  and  $0.03 \pm 0.01\%$  of the total conidia to which they were exposed, respectively. For each life stage

the whole body acquisition rate decreased significantly as the application rate increased (nymphs:  $F_{[2,13]} = 55.0$ ,  $P < 0.0001$ ; adult females:  $F_{[3,12]} = 22.3$ ,  $P < 0.0001$ ), (Fig. 5). The acquisition rates among the body parts varied significantly as a function of both application rate (nymphs:  $F_{[2,13]} = 14.6$ ,  $P = 0.0005$ ; adult females:

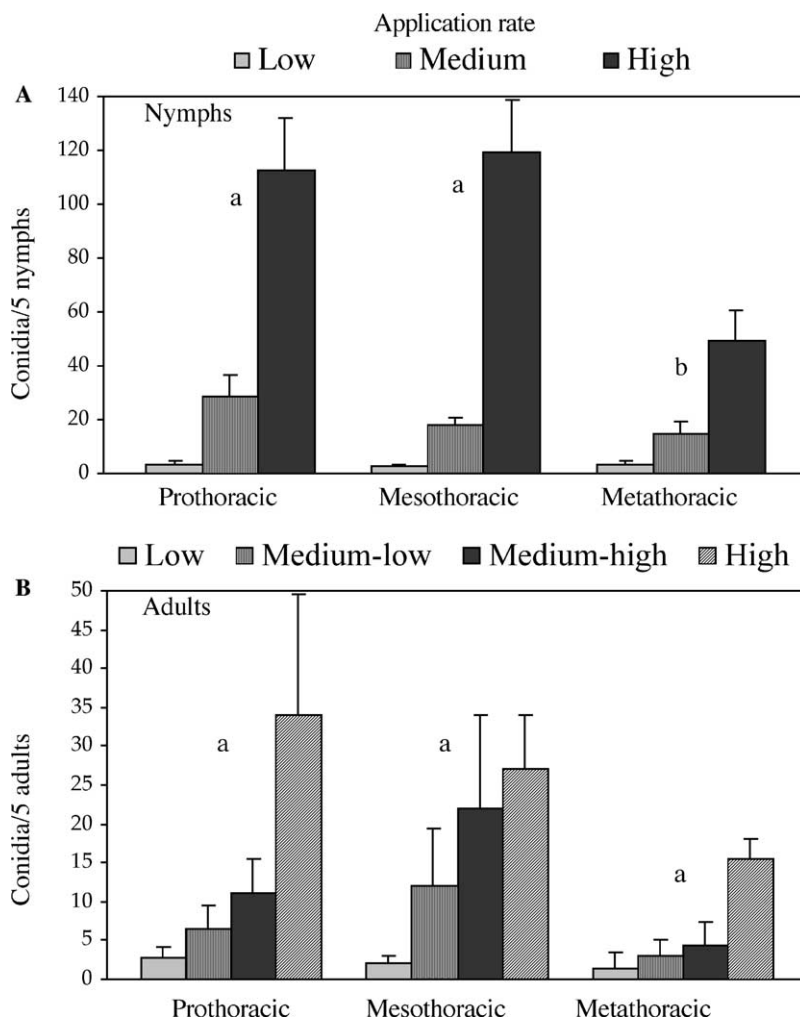


Fig. 3. Conidia on legs per five second instar nymphs or per five adult female western flower thrips exposed 24 h to bean leaf disks treated with *B. bassiana* at three or four application rates (5, 63, and 974 conidia/mm<sup>2</sup>; 11, 69, 285, and 1880 conidia/mm<sup>2</sup>, respectively); bars represent standard errors. Means (across application rates) with the same letter are not significantly different (Tukey's HSD test;  $\alpha = 0.05$ ).

$F_{[3,12]} = 16.5$ ,  $P < 0.0001$ ), and body part (nymphs:  $F_{[4,10]} = 3.7$ ,  $P = 0.04$ ; adult females:  $F_{[5,8]} = 53.0$ ,  $P < 0.0001$ ). The interaction between application rate and body part was insignificant for both nymphs and adult females (nymphs:  $F_{[8,22]} = 1.2$ ,  $P = 0.33$ ; adult females:  $F_{[15,30]} = 1.7$ ,  $P = 0.34$ ).

Acquisition rates per unit surface area (0.10 mm<sup>2</sup>) were calculated for both second instars and adult females, and the effects of application rate and body part were investigated. There was a highly significant effect of application rate (nymphs:  $F_{[2,13]} = 25.4$ ,  $P < 0.0001$ ; adult females:  $F_{[3,12]} = 46.3$ ,  $P < 0.0001$ ) and body part (nymphs:  $F_{[4,10]} = 17.7$ ,  $P = 0.0002$ ; adult females:  $F_{[5,8]} = 40.8$ ,  $P < 0.0001$ ) (Fig. 5). Again, a strong inverse relationship between application rate and acquisition rate was noted. There was a marginally significant interaction (application rate  $\times$  body part) for second instars (nymphs:  $F_{[8,22]} = 2.4$ ,  $P = 0.05$ ) but not for adults (adult females:  $F_{[15,30]} = 1.8$ ,  $P = 40.08$ ).

#### 4. Discussion

Second-instar thrips exposed to bean leaf disks with dry *B. bassiana* residues did not acquire significantly different numbers of conidia on the whole body or any specific body parts as a function of fungus formulation. There were no statistical differences in the LD<sub>50</sub>s, LC<sub>50</sub>s, or the slopes of the probit regression lines among the different formulations (Table 2 and Fig. 1). The LD<sub>50</sub> reported here is an estimate of the actual number of conidia per insect that are required to cause 50% mortality. Estimates of LD<sub>50</sub> values reported as conidia/insect have been reported for only a few insects. Typically, counts of conidia on insects are difficult to obtain and require time consuming and expensive techniques like scanning electron or fluorescence microscopy. Researchers have relied on reporting lethal exposures to fungal pathogens in units of conidia or colony forming units (CFUs)/ml of suspension, or conidia/unit area of substrate.

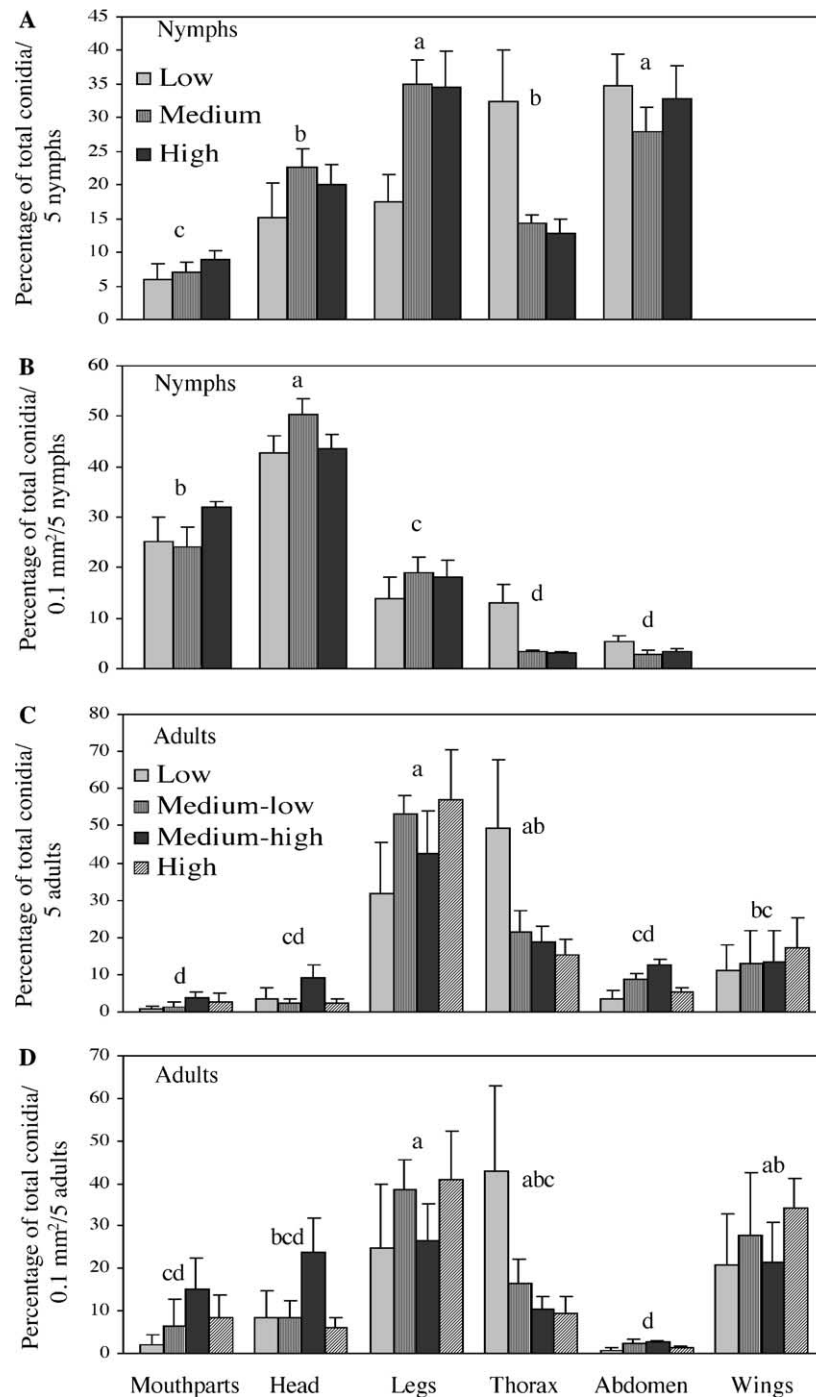


Fig. 4. Distributions of conidia acquired per five second instar or five adult female western flower thrips exposed 24 h to bean leaf disks treated with *B. bassiana* at three or four application rates (5, 63, and 974 conidia/mm<sup>2</sup>; 11, 69, 285, and 1880 conidia/mm<sup>2</sup>, respectively). Distributions expressed as the percentage of conidia observed per body part (A, C) and per unit surface area of each body part (B, D), relative to the whole-body total; bars represent standard errors. Means (across application rates) with the same letter are not significantly different (Tukey's HSD test;  $\alpha = 0.05$ ).

The finding of no formulation effect was unexpected in lieu of reports by numerous researchers (Bateman et al., 1993; Fargues et al., 1997; Ibrahim et al., 1999; Inglis et al., 1996; Jenkins and Thomas, 1996; Milner et al., 1997; Prior et al., 1988; Wraight and Ramos, 2002). These researchers investigated the effects of different

carriers, oils versus water, against a range of insects using a wide variety of exposure methods including direct spray application, micro-pipetting conidia onto the insect, feeding treated foliage to insects, and exposing insects to treated foliage. In the majority of the experiments conducted, conidia formulated in oils were more

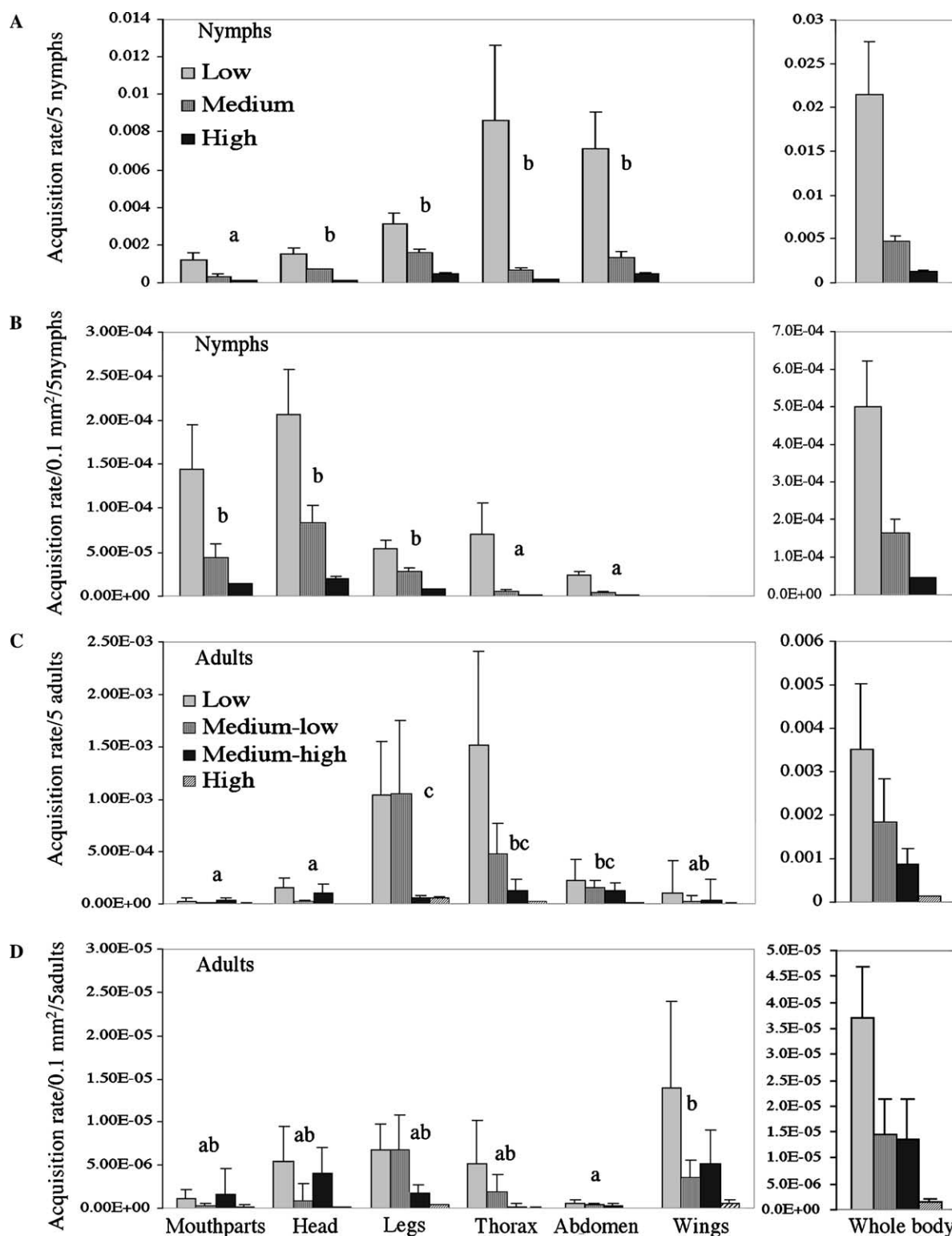


Fig. 5. Conidial acquisition rates per specified body part (A, C) and per unit surface area of each body part (B, D) per five second instar or five adult-female thrips exposed 24 h to bean leaf disks treated at three or four application rates (5, 63, and 974 conidia/mm<sup>2</sup>; 11, 69, 285, and 1880 conidia/mm<sup>2</sup>, respectively). Acquisition rate is expressed as conidia acquired/total conidia to which thrips were exposed on treated leaf disks. Bars represent standard errors; means (across application rates) with the same letter are not significantly different (Bonferroni-adjusted t tests;  $\alpha = 0.05$ ).

efficacious, as measured by speed of kill. However, the magnitude of the effects of formulation varied greatly across studies and typically was not large.

The results reported herein suggest that the oil carrier used in the commercial formulation did not enhance adhesion of conidia to either the leaf cuticle or the insect

cuticle. The oil droplets encapsulating the conidia in the EO formulation may have been absorbed and dissipated by the waxy leaf cuticle, leaving the conidia essentially unformulated on the leaf surface as suggested by Inglis et al. (1995), who noted rapid loss of UV resistance after oil-formulated *B. bassiana* conidia were applied to grass leaves.

Comparisons of the lethal dose of the WP formulation against second-instar thrips and adult-female thrips suggest that the adult thrips were substantially more susceptible than the second instars ( $LD_{50}$  of ca. 5 vs 50 conidia per insect, respectively). Presumably the greater infectivity against adult thrips is because they do not molt and are thus exposed to conidia for a longer period of time compared to second-instar nymphs. Duration of the second instar is 3.5 days at 25 °C (Robb and Parrella, 1991), and the nymphs are highly susceptible to infection by *B. bassiana* for only ca. the first 24 h of the instar (Ugine, 2004). This shortened window of infection time for second instars may have important implications when attempting to manage thrips populations of mixed ages as they occur in agricultural settings.

One unexpected result of this study was the difference in the number of conidia acquired by the whole bodies of adult females vs second-instar nymphs. Second-instar thrips acquired 2 times more conidia than adult females despite the obvious difference in surface areas. This difference could possibly be explained by differences in behavior and degrees of mobility/activity of the two life stages. Nymphal thrips are in a stage of maximum potential growth rate and need to actively feed as much as possible. Thus their movement from the treated leaf disks is limited, especially compared to winged adult thrips. In contrast, adult females actively search for a mate and carry eggs that need to mature before oviposition; successful egg maturation is enhanced by access to a protein-rich food source, like pollen. Although adults undoubtedly fed on the treated leaf discs, their searching activity could have removed them from the sprayed leaves and limited their exposure to conidia. Another possible explanation for the difference in conidia acquisition is the characteristic grooming behavior of adult thrips pictured by Ellington (1980). Before flight, the wings are brushed repeatedly over the body potentially removing conidia or transferring them from ventral to dorsal surfaces or vice versa.

The distribution of conidia on the bodies of second-instar thrips was similar to that of adult thrips. For each life stage, the legs and thoraces acquired the largest percentages of conidia, and the mouthparts acquired the smallest percentage. The percentage of conidia acquired by the abdomens of second instars was also large (not significantly different than that of the legs), whereas for adults the abdomen acquired one of the smallest percentages. It is not surprising that the legs of each life

stage acquired a large percentage of conidia considering that the legs are in constant contact with the treated surface. It is puzzling, however, that the mouthparts, another body part that is likely to be in frequent contact with the treated substrate, acquired such a small percentage of conidia. The low percentage of conidia found on the abdomens of adults, compared to seconds, could be due to a shielding or protective effect of the wings, which are held over the abdomen when not in use. Because thrips can be located between treated leaves, conidia surround the thrips in the assay chamber. Both the dorsal and ventral surfaces of thrips could come into contact with conidia, thus explaining the presence of conidia on the wings. Also, adult thrips of many species are known to comb their wings over their bodies before flight to loosen the many hairs on the wings. This behavior has the potential to move spores from the body onto the wings.

The rate of conidia acquisition on the whole bodies of second-instar and adult-female thrips decreased significantly as the density of conidia on the leaf disk surface increased. At application rates ranging from 5–973 to 11–1880 conidia/mm<sup>2</sup> (application rates standardized to 100 conidia/mm<sup>2</sup>), in trials of nymphs and adults, respectively, nymphs acquired between 17–230 conidia/100 conidia/mm<sup>2</sup> applied and adult females acquired 2–47 conidia/100 conidia/mm<sup>2</sup>. The greatest numbers of conidia acquired/100 conidia/mm<sup>2</sup>, by nymphs and adults (230 and 47 conidia) were acquired at the lowest conidial densities (5 and 11 conidia/mm<sup>2</sup>, respectively). There are several possible hypotheses for such a relationship. As the density of conidia on the leaf substrate increases, thrips acquire an increasing dose. If there exists a threshold number of conidia that can become attached to the body before attachment points become saturated, the rate of further acquisition would diminish. However, during the visual inspections of the thrips, it never appeared that any body parts were saturated with conidia. Alternatively, thrips may avoid treated surfaces, or arrest movement in the presence of substrates treated with high numbers of conidia, or become increasingly agitated by conidial adherence or hyphal penetration, and leave the treated surface. In our bioassays, thrips repelled by the spray residues could have opted to feed on the untreated, axial surfaces of the leaf disks. Lastly, as conidial density on treated leaves increases, it is likely that the incidence of conidial clumping also increases and it may be that conidia are not acquired by thrips as efficiently when clumped. Large aggregates of conidia may not readily dislodge from the substrate and individual conidia may not be readily separated from an aggregation. Given that all of the formulations of *B. bassiana* tested elicited the same trend of decreasing acquisition rate with increasing conidia density (data not presented), it is likely that this phenomenon is not caused by a formulation ingredient.

Lower probit slopes for the LD regressions compared to the LC regressions, which were most evident in the tests of adults, supports the finding of an inverse relationship between dose and acquisition rate. In the regression analyses, it is assumed that the spore concentrations on the leaf disks are a reliable relative measure of the doses applied against the insects (in any model 1 regression, dose is assumed to have been measured without error). If the conidia repel the insects or somehow become more difficult to pick-up at higher concentrations, estimates of spore concentrations may be biased. At the higher concentrations, doses are overestimated, resulting in underestimation of the regression coefficient (slope). This result suggests that the low slopes often obtained in fungal assays may arise, at least in part, as an artifact of unequal rates of dose acquisition at low vs high application rates. This warrants further investigation.

A growing body of literature is reporting that insects can detect and avoid both conidia and mycelium of hyphomycete fungi. German cockroach nymphs, *Blattella germanica*, often cannibalize other dead roaches. Kaakeh et al. (1996) reported that roaches killed by the fungus *Metarhizium anisopliae* and covered in mycelium were not cannibalized, which they concluded was an avoidance response. Several studies have demonstrated that *M. anisopliae* conidia (Milner and Staples, 1996), *B. bassiana* conidia, and non-viable 'dead' *M. anisopliae* are repellent to termites (Staples and Milner, 2000), and that termites wall off sections of fungus-contaminated mounds (Milner, 2000). Rath (2000) provides a more detailed review of termite behavior in response to entomopathogenic fungi. These instances of insects avoiding sources of entomopathogenic fungal inoculum support a hypothesis of avoidance, but further investigation is needed to determine the mechanism behind the thrips phenomenon. Further study of this phenomenon (decreased rate of dose acquisition with increasing application rate) could identify ways to improve mycoinsecticide efficacy.

## Acknowledgments

This research was funded in part through a Specific Cooperative Agreement between the USDA/ARS Plant Protection Research Unit and the Cornell University Department of Entomology, Ithaca, NY (SCA #58-1097-9-033) funded by the USDA/ARS, as part of the Floriculture and Nursery Research Initiative.

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